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Pineal Gland: Dibutyryl Cyclic Adenosine Monophosphate Stimulation of Labeled Melatonin Production

Abstract. *In organ cultures of intact rat pineal glands, $N^6O^{2'}$-dibutyryl adenosine 3',5'-monophosphate stimulates the conversion of tritiated trytophan to tritiated melatonin, as does* L-*norepinephrine. Potential sites of stimulation of melatonin production by dibutyryl cyclic adenosine monophosphate are discussed, based on observations that the dibutyryl analog also stimulates the conversion of serotonin labeled with carbon-14 to carbon-14-labeled melatonin without altering hydroxyindole-*O*-methyl transferase activity or intracellular accumulation of serotonin labeled with carbon-14.*

Melatonin is synthesized from serotonin by *O*-methylation of the intermediate *N*-acetylserotonin (*1*). In mammals this conversion takes place only in the pineal gland, because of the presence of the unique enzyme hydroxyindole-*O*-methyl transferase (*2*). Norepinephrine stimulates the conversion of labeled tryptophan (*3*) and serotonin (*4*) to melatonin by pineal glands in two different organ culture systems. Weiss and Costa (*5*) reported that norepinephrine also stimulated the activity of adenyl cyclase in homogenates of pineal glands, implying that this monoamine may elevate the concentration of adenosine 3',5'-monophosphate (cyclic AMP) in the gland. The role of cyclic AMP as an intermediate or "second messenger" in many hormone-target organ systems is becoming increasingly apparent (*6*), and we investigated whether the stimulatory effects of norepinephrine on melatonin synthesis in the pineal gland would be mimicked by cyclic AMP.

We tested the hypothesis that cyclic AMP may directly stimulate melatonin production using an organ culture system (*7*). Intact pineal glands were obtained from female Osborne-Mendel rats (150 to 170 g) and incubated for 24 hours in 0.5 ml of chemically defined media (*8*) supplemented with serum albumin (fraction V, Pentex, 1 mg/ml). Labeled melatonin formed from uniformly labeled [^{3}H]tryptophan or [^{14}C]serotonin (Amersham Searle) was extracted into 8.0 ml of chloroform from 0.05 to 0.10 ml of media added to 2 ml of 0.5*M* sodium borate buffer (*p*H 10). Chloroform extracts were washed once with 2 ml of this buffer and twice with 2.0 ml of 0.1*N* HCl or 1*N* HCl in the case of [^{14}C]serotonin incubations. Ninety to ninety-five percent of added melatonin was extracted by this procedure. Samples of the chloroform extracts were evaporated to dryness, and the radioactivity was measured in a liquid scintillation spectrometer. To verify the identity of the radioactive product in the chloroform extract, portions of all sample extractions within one treatment group were evaporated in an N_2 atmosphere, redissolved in a solution of alcohol and 1*N* HCl containing standard carriers (Regis Chemical), and analyzed by thin-layer chromatography (*9*). Seventy-five to ninety-five percent of the radioactivity extracted from the incubation media of pineal glands treated with dibutyryl cyclic AMP (2×10^{-4} to $8 \times 10^{-4}M$) chromatographed with authentic melatonin. The radioactivity extracted from the media of untreated and unstimulated glands was too small to allow analysis by thin-layer chromatography.

We examined the effects of L-norepinephrine (Regis Chemical Company), cyclic AMP, and dibutyryl cyclic AMP (potassium salt, Lot No. 840092; Calbiochem Corporation) on the conversion of [^{3}H]tryptophan (Table 1). Norepinephrine stimulates the conversion of [^{3}H]tryptophan to [^{3}H]melatonin (*3*). Cyclic AMP ($10^{-3}M$) had no influence on this conversion. However, dibutyryl cyclic AMP, a substance reported to have effects similar to cyclic AMP in certain tissues (*6*), produced a marked stimulation at concentrations greater than $2 \times 10^{-4}M$ (Table 1). The greater effect of dibutyryl cyclic AMP was probably a result of slower degradation by phosphodiesterase and of more rapid entry into the cell, relative to the parent compound, or a combination of these factors (*10*). The similar effects of norepinephrine and dibutyryl cyclic AMP on tryptophan conversion to melatonin, together with the known stimulatory effects of norepinephrine on pineal gland adenyl cyclase (*5*), is consistent with the idea that one mechanism by which norepinephrine may stimulate melatonin production involves elevation of tissue concentrations of cyclic AMP.

We next investigated the mechanism by which dibutyryl cyclic AMP stimulated the conversion of [^{3}H]tryptophan by studying whether stimulation in the metabolic pathway occurred before or after the synthesis of serotonin. Dibutyryl cyclic AMP stimulated the conversion of substrate concentrations ($10^{-4}M$) of [^{14}C]serotonin (Table 2), an indication that dibutyryl cyclic AMP, as L-norepinephrine (*4*), acts at a metabolic step after the synthesis of serotonin. Any mechanism which stimulated the endogenous formation of unlabeled serotonin would have been expected to decrease conversion of labeled exogenous serotonin to labeled melatonin by diluting the radioactive serotonin.

The amounts of [^{14}C]serotonin con-

Table 1. Effects of dibutyryl cyclic AMP, and L-norepinephrine on conversion of [^{3}H]tryptophan to labeled melatonin in rat pineal glands. The glands were incubated for 24 hours with [^{3}H]tryptophan (5.2 μc/ml, 26 μc/μm). One gland was incubated in 0.5 ml of media. Data on [^{3}H]melatonin are based on groups of three or four determinations. Statistical analysis was performed by Student's *t*-test.

Treatment	Concentration (M)	N	[^{3}H]Tryptophan converted to labeled melatonin (pmole)
Control		4	29 ± 36
L-Norepinephrine	1.0×10^{-5}	4	231 ± 40*
Cyclic AMP	1.0×10^{-3}	3	59 ± 48
Dibutyryl cyclic AMP	7.4×10^{-7}	4	73 ± 25
Dibutyryl cyclic AMP	3.1×10^{-6}	4	84 ± 28
Dibutyryl cyclic AMP	1.3×10^{-5}	3	117 ± 56
Dibutyryl cyclic AMP	5.0×10^{-5}	4	121 ± 25
Dibutyryl cyclic AMP	2.0×10^{-4}	4	544 ± 81†
Dibutyryl cyclic AMP	8.0×10^{-4}	4	620 ± 168*

* $P < .02$. † $P < .01$.

verted to [^{14}C]melatonin was less than the amount of [^{3}H]tryptophan converted to [^{3}H]melatonin. This would be anticipated from the experimental design. In studies with [^{14}C]serotonin in which the concentration of serotonin in the media was approximately $10^{-4}M$, there were two precursor sources available for acetylation. One source was endogenous serotonin synthesized continuously from tryptophan in the media and the other was [^{14}C]serotonin. Stimulation of either acetylation or *O*-methylation would have removed serotonin from both these sources, and melatonin of a lower specific activity than [^{14}C]-serotonin would be produced.

The alternate possibility was that dibutyryl cyclic AMP could have increased [^{14}C]melatonin production by increasing intracellular transport or accumulation of extracellular radiolabeled serotonin. This appeared unlikely because dibutyryl cyclic AMP did not alter [^{14}C]serotonin accumulation in glands after 24 hours of incubation (Table 2).

Hydroxyindole-*O*-methyl—transferase (HIOMT) has been thought to be rate limiting in melatonin synthesis (*1*). When we assayed HIOMT activity (*11*), in glands after 24 hours of incubation, no effect of dibutyryl cyclic AMP was detected (Table 2) (*12*). In addition, neither dibutyryl cyclic AMP nor cyclic AMP altered the activity of HIOMT when added directly to homogenates of unincubated pineal glands, suggesting that these compounds did not directly activate HIOMT. These studies did not rule out the possibility that cellular HIOMT activity may be controlled by activators produced in response to dibutyryl cyclic AMP, and, in such a case, the effect of activators might be lost by the 1500-fold dilution of the crude homogenate prepared for HIOMT assay.

We found the activity of HIOMT in broken-cell homogenates of control and stimulated glands to be about 100 to 120 pmole of melatonin produced per hour. However, even in maximally stimulated glands the average approximated rate of melatonin production from [^{3}H]tryptophan or [^{14}C]serotonin did not appear to be greater than 25 pmole/hr. In unstimulated glands, this rate was less than 3 pmole/hr. Although HIOMT has been thought to be the rate-limiting enzyme in melatonin synthesis (*1*), these observations suggest that dibutyryl cyclic AMP may control melatonin production in this experimental model through another mechanism. Dibutyryl cyclic AMP may primarily stimulate the acetylation of serotonin and control melatonin production through substrate availability for HIOMT.

Table 2. Effects of dibutyryl cyclic AMP ($8 \times 10^{-4}M$) on conversion of [^{14}C]serotonin and [^{3}H]tryptophan to labeled melatonin, on HIOMT activity, and on content of [^{14}C]serotonin in pineal glands incubated for 24 hours. In groups 1 and 2, two glands were incubated in 0.5 ml of media with [^{3}H]tryptophan (7.8 μc/ml, 39 μc/μm). Data on [^{3}H]melatonin are based on groups of four determinations and HIOMT data on groups of eight individual determinations. In groups 3 and 4, single glands were incubated in 0.5 ml of media containing [^{14}C]serotonin (3.0 μc/ml, 56 μc/μm). The HIOMT activity is expressed as the picomoles of melatonin (-*O*-methyl-^{14}C) formed per hour per gland (*11*). Statistical analysis was performed by Student's *t*-test.

Group	N	[^{3}H]Tryptophan converted to [^{3}H]melatonin (pmole/gland)	HIOMT activity	[^{14}C]Serotonin converted to [^{14}C]melatonin (pmole/gland)	Total pineal gland [^{14}C]serotonin (ng/gland)
1. Control	8	88 ± 8.5	123 ± 18		
2. Dibutyryl cyclic AMP	8	693 ± 134.0*	104 ± 20		
3. Control	4			74 ± 20	32 ± 3.9
4. Dibutyryl cyclic AMP	4			220 ± 41*	32 ± 6.5

* $P < .01$.

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11. J. Axelrod and H. Weissbach, *J. Biol. Chem.* **236**, 211 (1961); D. C. Klein and S. Lines, *Endocrinology* **84**, 1523 (1969). The HIOMT reaction mixture includes *S*-adenosyl methionine ($3 \times 10^{-5}M$), *N*-acetylserotonin ($10^{-4}M$), 100 nc of [^{14}C-methyl] *S*-adenosyl methionine (Amersham Searle), and 40 percent of the homogenate of one gland in a total of 0.3 ml of 0.5*M* sodium phosphate buffer (*p*H 7.9).
12. During review of this paper, Shein and Wurtman [*Science* **166**, 519 (1969)] reported that dibutyryl cyclic AMP increases the formation of [^{14}C]melatonin and [^{14}C]serotonin from [^{14}C]tryptophan. Our results agree with these because to increase synthesis of melatonin from tryptophan the synthesis of (the intermediate) serotonin may be increased. However, studies using the inhibitor of serotonin synthesis *p*-chlorophenylalanine (*p*CPA) seem to indicate that the effect of dibutyryl cyclic AMP in stimulating melatonin production is not dependent on prior elevation of serotonin production. We have found that whereas *p*CPA (1.0 m*M*) inhibits the effect of dibutyryl cyclic AMP on conversion of [^{3}H]tryptophan to [^{3}H]melatonin to 35 ± 20 percent of the normal stimulation, the effect of dibutyryl cyclic AMP on [^{14}C]serotonin conversion to melatonin is not reduced by *p*CPA, but is slightly enhanced (125 ± 20 percent). This suggests that a specific site of action of dibutyryl cyclic AMP involved in stimulating melatonin production is at a metabolic step that does not depend on the new synthesis of serotonin, and will take place when an exogenous source of serotonin is provided, as in the experiments presented here.

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